



# Interindividual Differences in Human In Vitro Intestinal Microbial Conversion of Green Tea (-)-Epigallocatechin-3-O-Gallate and Consequences for Activation of Nrf2 Mediated Gene Expression

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**Scope:** An in vitro faecal incubation model combined with reporter gene assay based testing strategy is developed to characterize interindividual differences in the gut microbial conversion of (-)-epigallocatechin-3-O-gallate (EGCG) and its consequences for potential activation of Nrf2-mediated gene expression.

**Method & Results:** Anaerobic human faecal incubations are performed to characterize the microbial metabolism of EGCG including interindividual variability. EGCG derived intestinal microbial metabolite patterns show substantial interindividual differences that are correlated to relative microbial abundances determined by 16S rRNA sequencing. Results obtained show the time-dependent formation of gallic acid, pyrogallol, phenylpropane-2-ols, phenyl- $\gamma$ -valerolactones, and 5-(3',5'-dihydroxyphenyl)valeric acid as the major metabolites, with substantial interindividual differences. The activity of the formed metabolites in the activation of EpRE-mediated gene expression is tested by EpRE-LUX reporter gene assay. In contrast to EGCG, at low micromolar concentrations, especially gallic acid, pyrogallol, and catechol induce significant activity in the EpRE-LUX assay.

**Conclusions:** Given these results and taking the level of formation into account, it is concluded that especially gallic acid and pyrogallol contribute to the EpRE-mediated beneficial effects of EGCG. The interindividual differences in the formation may result in interindividual differences in the beneficial effects of EGCG and green tea consumption.

## 1. Introduction

Green tea is rich in some natural polyphenolic compounds, especially catechins including (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-O-gallate (ECG), and (-)-epigallocatechin-3-O-gallate (EGCG), which comprise 30–42% (w/w) of the solids in green tea infusion.<sup>[1]</sup> Among these components, EGCG is the most abundant polyphenol, making up more than 50% of the total catechin constituents.<sup>[2]</sup> Regular consumption of green tea is often considered to have protective effects against cardiovascular diseases and certain forms of cancer.<sup>[3]</sup> The mode of action underlying these potential beneficial health effects may in part be related to activation of the Keap1/Nrf2 (Kelch ECH associating protein 1/nuclear factor erythroid 2-related factor 2) system, a thiol-based sensor-effector apparatus that is involved in redox homeostasis.<sup>[4]</sup> Once Nrf2 is released from its complex with Keap1, it will translocate from

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cytoplasm to nucleus where its binding to the electrophile-responsive element (EpRE) with small Maf proteins, results in the up-regulation of EpRE-mediated gene expression.<sup>[4]</sup> This EpRE-mediated gene expression is considered to play an essential role in the prevention of various adverse health effects.<sup>[5,6]</sup> EGCG was reported to be able to increase both mRNA and protein levels of Nrf2 in mice. The upregulation and nuclear translocation of Nrf2 induce the expression of EpRE-regulated conjugation enzymes including for example UDP-glucuronosyltransferases (UGTs). The increased UGT activity facilitates conjugation, detoxification, and excretion of toxic electrophiles able to generate protein and DNA damage, thereby contributing to prevention of a range of adverse health effects including carcinogenicity. It was reported, for example, that EGCG could inhibit the growth and metastasis of orthotopic colon cancer implants in nude mice in a dose-dependent manner.<sup>[3,7]</sup>

Several green tea catechins have been reported to activate Keap1/Nrf2 in in vitro models at high  $\mu\text{M}$  concentrations.<sup>[8,9]</sup> However, in vivo the bioavailability and plasma concentrations of catechins are usually quite low.<sup>[10]</sup> After intake of green tea infusion, only a small proportion of the ingested catechins is absorbed by the small intestine, while a large fraction reaches the large intestine where the catechins are metabolized by colonic microbiota. It has been estimated that only 5–10% of ingested dietary polyphenols are absorbed in the small intestine, while substantial amounts reach the colon where they are intensively degraded by microbiota into a diversity of bioactive phenols, phenyl- $\gamma$ -valerolactones, and phenolic acids that are subsequently absorbed.<sup>[11–13]</sup> To characterize the colonic microbial metabolite pattern of EGCG, in the present study an in vitro anaerobic incubation model was applied. **Figure 1** provides an overview of the tentative metabolic pathways for EGCG as derived from literature data,<sup>[3,13]</sup> and results we obtained on intestinal microbial metabolism from the present study. Given the low systemic concentrations of EGCG (and/or EGCG conjugates) and the (in some cases) even higher systemic concentrations of microbial metabolites, it can be hypothesized that their microbial metabolites may contribute to (some of) their health-promoting effects. For example, one of the important microflora-derived catechin metabolites, 5-(3',4'-dihydroxyphenyl)- $\gamma$ -valerolactone (3,4-diHPV), was reported to be able to inhibit NO production and iNOS expression in RAW 264.7 cells, suggesting potential anti-inflammatory activity.<sup>[14]</sup>

There are trillions of microbes inhabiting the human intestine and the host intestinal microbiome can be remarkably variable across individuals due to the differences in age, gender, dietary and lifestyle, etc.<sup>[15]</sup> Thus, the microbial metabolism of EGCG could also vary between individuals. As a result, interindividual differences in the levels of potential health promoting metabolites resulting from EGCG or green tea consumption may also exist.

The aim of the present study was to characterize the human intestinal microbiota-mediated conversion of EGCG, including the interindividual variability, using an in vitro testing strategy, and to identify the activity of the formed metabolites in the activation of EpRE-mediated gene expression. To this end, the time-dependent metabolite pattern of EGCG was quantified in in vitro human faecal incubations. Faecal microbial taxonomy profiles were characterized by 16S rRNA analysis and bacterial loads quantified by

quantitative PCR (qPCR), which allowed the identification of correlations between taxon abundances and formation of colonic metabolites. Furthermore, EpRE-mediated Nrf2 activation by the formed metabolites was characterized using an EpRE-LUX reporter cell line previously developed in our lab.<sup>[16]</sup>

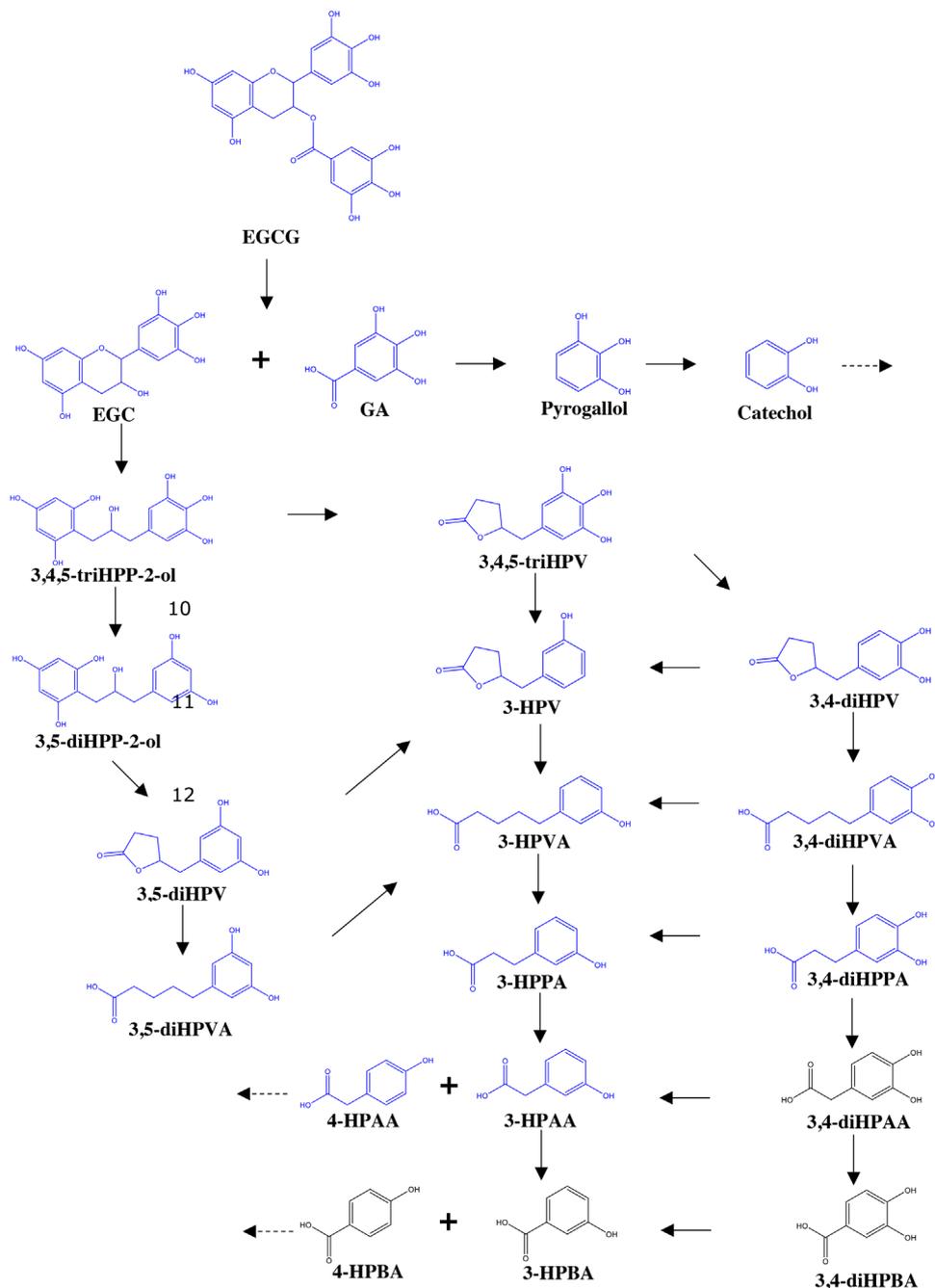
## 2. Results

### 2.1. LC-TQ-MS and LC-TOF-MS Based Identification of Colonic Metabolites of EGCG

**Figure 2** presents the time-dependent formation of EGCG metabolites formed in anaerobic incubations with a pooled human faecal sample. Concentrations of metabolites formed were corrected for the amounts detected in corresponding negative controls incubated without adding EGCG. Six polyphenols were detected in the negative controls but in limited amounts (<11.5% of the total mass recovery, Figure S2, Supporting Information). The chemical structures of the metabolites detected by liquid chromatograph triple quadrupole mass spectrometry (LC-TQ-MS) and liquid chromatograph time-of-flight mass spectrometry (LC-TOF-MS) are highlighted in blue color in **Figure 1**.

The metabolic profiles observed corroborate that the first step of EGCG bioconversion consists of its hydrolysis to produce gallic acid and EGC. During the first 2 h incubation, gallic acid formation reached a plateau at  $17.0 \mu\text{M}$ , accounting for 33.9% (mol per mol) of the EGCG added to the incubation. Upon further incubation, gallic acid levels gradually decreased with only  $0.3 \mu\text{M}$  detected at 6 h, representing 0.6% of the original EGCG equivalents. Further metabolism of gallic acid proceeds by its decarboxylation to generate pyrogallol. Pyrogallol peaked at 4 h incubation with  $15.9 \mu\text{M}$ , amounting to 31.7% of the original EGCG equivalents. Additionally, a limited amount of catechol was detected resulting from the further conversion of pyrogallol, pointing at swift further degradation of catechol preventing its accumulation.

The second metabolite resulting from the initial EGCG hydrolysis, EGC, was detected at concentrations that were at most  $8.2 \mu\text{M}$  at 1 h, accounting for 16.4% of the original EGCG equivalents. After 3 h incubation, EGC was almost fully degraded with only  $0.8 \mu\text{M}$  (1.6% EGCG equivalents) remaining at 3 h. EGC underwent reductive cleavage in the C-ring producing 1-(3',4',5'-trihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-2-propanol (3,4,5-triHPP-2-ol). This intermediate maximized at 2 h incubation, amounting to  $11.0 \mu\text{M}$ , (22.1% of the original EGCG equivalents). Subsequently, 3,4,5-triHPP-2-ol can be degraded through two major pathways, dehydroxylation to generate 1-(3',5'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-2-propanol (3,5-diHPP-2-ol) or A-ring fission to form 4-H-triHPVA, which was a transient metabolite that appeared to isomerize to 3,4,5-triHPV. The first compound, 3,5-diHPP-2-ol, further converted into 5-(3',5'-dihydroxyphenyl)- $\gamma$ -valerolactone (3,5-diHPV) which was one of the main intermediates, detected at 1 h in still small amounts ( $0.3 \mu\text{M}$ , 0.6% EGCG equivalents) but increasing until it peaked at 6 h at  $17.9 \mu\text{M}$ , (35.7% EGCG equivalents). After 24 h incubation, the two phenyl- $\gamma$ -valerolactones were almost fully converted into 5-(3',5'-dihydroxyphenyl)valeric acid (3,5-diHPVA) and 5-(3'-hydroxyphenyl)valeric acid (3-HPVA), respectively, that accounted for 61.0% ( $30.5 \mu\text{M}$ ) and 23.4% ( $11.7 \mu\text{M}$ ) of the originally added amount of EGCG. In addition to these major



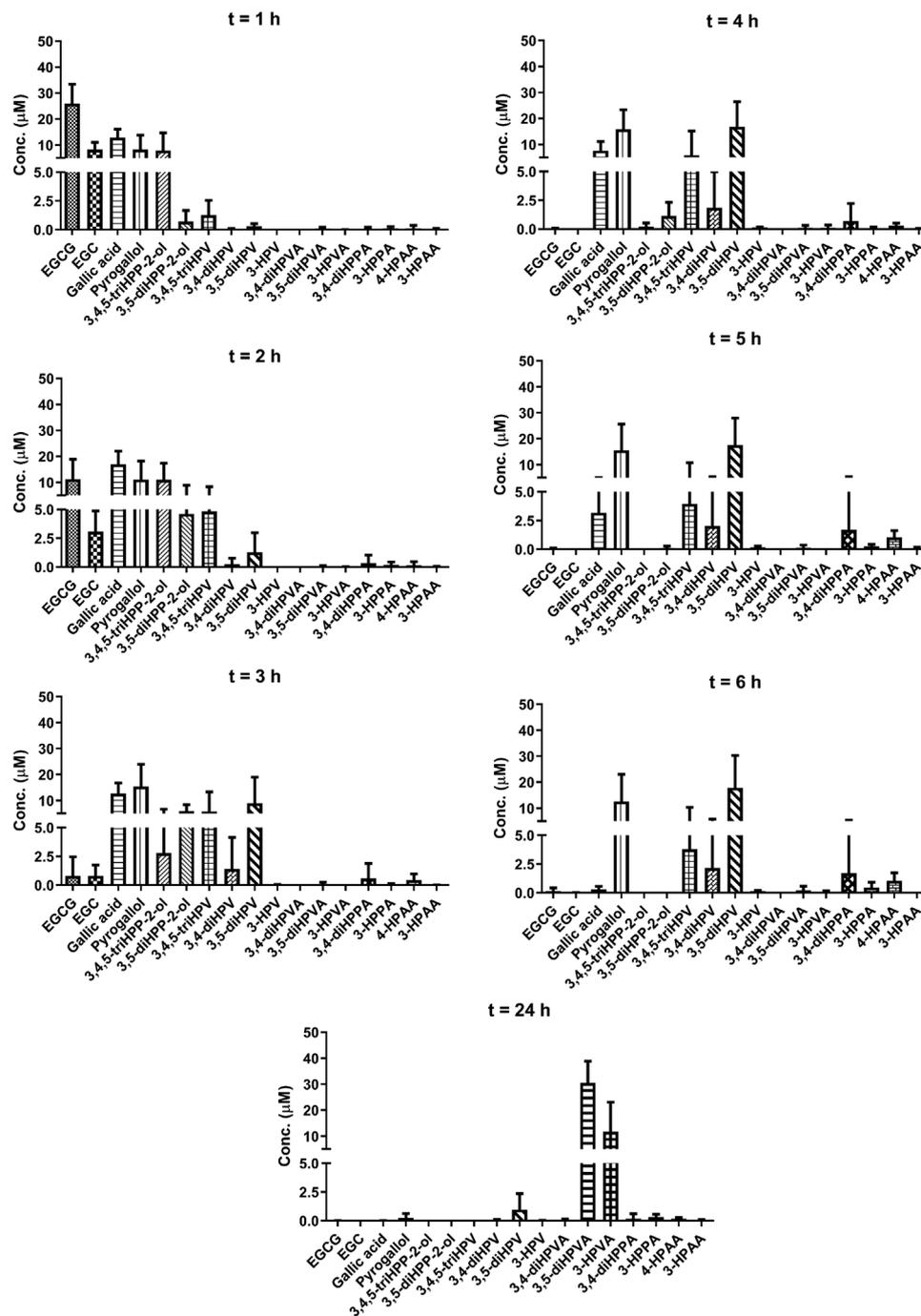
**Figure 1.** Tentative pathways in the human intestinal microbial metabolism of EGCG. Structures in blue color were observed in the present study.

metabolites, small amounts of some phenol propionic acids and phenol acetic acids were also quantitatively detected in the incubations (Figure 2). **Figure 3A,B** shows the total molar mass recovery at different incubation timepoints for the gallic acid and EGC degradation routes, respectively. For the gallic acid degradation route, the total mass recovery started to decrease from 1 h onward, dropping from 99.8% at 1 h to 25.7% at 6 h and being almost undetectable at 24 h. This indicates that eventually pyrogallol is further degraded into metabolites that were not included in the present MS measurement. For the EGC degradation route, the total mass recovery also started to

decrease from 1 h (94.5%) onward but remained relatively high during the further incubation ( $\geq 57.5\%$ ).

## 2.2. Interindividual Differences of EGCG Bioconversion by Human Colonic Microbiota

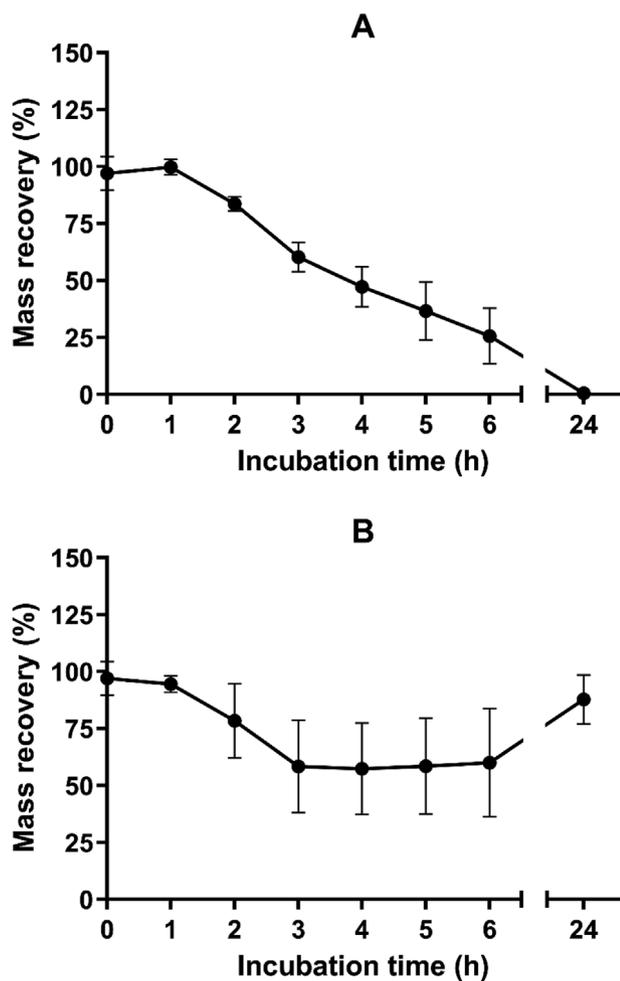
Based on the results obtained for the time-dependent *in vitro* microbial EGCG conversion in incubations with a pooled faecal sample, a period of 2 h was selected to quantify potential human interindividual differences in EGCG intestinal microbial



**Figure 2.** Quantitative time-dependent intestinal microbial metabolite patterns of EGCG formed during 24 h of *in vitro* anaerobic incubations with a pooled human faecal sample. The value above each graph indicates the time of incubation in hours. Results are shown as mean  $\pm$  standard deviation (SD) from three independent incubations. The same data are presented as time-dependent concentrations for each metabolite in Figure S9, Supporting Information.

metabolism. This time point was selected as still residual EGCG was present while at the same time substantial metabolite formation was observed. **Figure 4** shows the amount of residual EGCG and of seven main metabolites formed after incubating EGCG for 2 h with faecal suspensions from 14 human volunteers. Phenolic compounds quantified in different individuals in

the negative control are presented in Figure S3, Supporting Information. All phenolics were present in the negative controls at levels  $<12.8\%$  of total mass recovery with the exception of 3-(3'-hydroxyphenyl)propionic acid (82.5% of total mass recovery) in individual 3 and 3-(3',4'-dihydroxyphenyl)propionic acid (22.5% of total mass recovery) in individual 5. Meanwhile, substantial



**Figure 3.** Total mass recovery at different incubation timepoints for A) gallic acid degradation route and B) EGC degradation route. Results are shown as mean  $\pm$  standard error of mean (SEM) from three independent incubations.

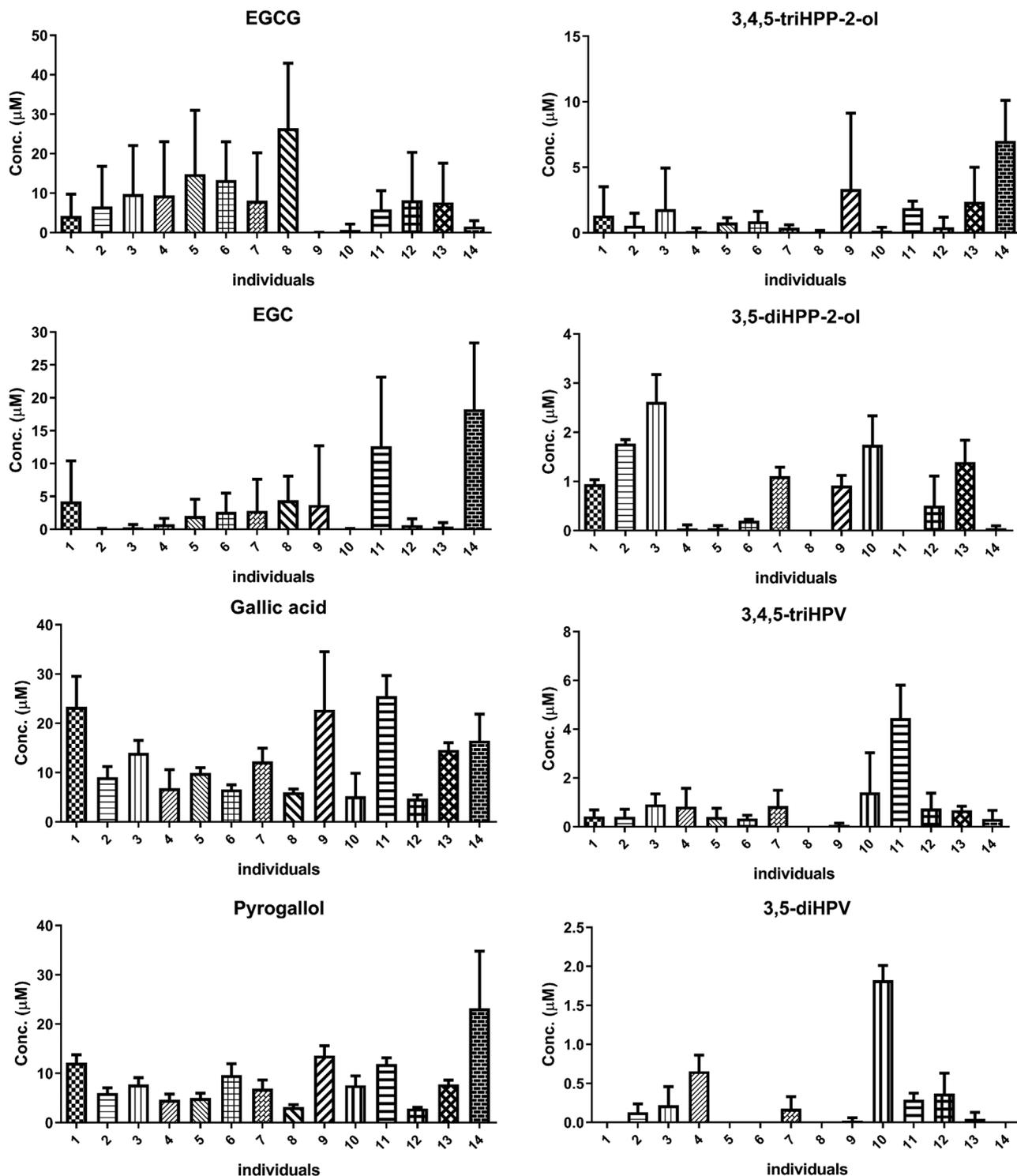
differences were observed both in terms of types and amounts of metabolites produced as well as in the level of EGCG depletion as shown in Figure 4. For example, upon 2 h incubation, less than  $1 \mu\text{M}$  (<1.3% of the initial concentration) of EGCG was detected in the incubations with faecal slurries from individuals 9 and 10, while  $26.5 \mu\text{M}$  (52.9% of the original amount) of EGCG was detected in the incubation with faecal slurry from individual 8. Likewise, substantial differences were also observed for the formation of major metabolites EGC, gallic acid, pyrogallol, 3,4,5-triHPP-2-ol, 3,5-diHPP-2-ol, 3,4,5-triHPV and 3,5-diHPV. For instance,  $23.2 \mu\text{M}$  (46.3% of original EGCG equivalents) of pyrogallol was quantified after 2 h incubation with faecal slurry from individual 14, while only  $2.8 \mu\text{M}$  (5.7% of original EGCG equivalents) of pyrogallol was detected in the incubation with faecal slurry from individual 12. Besides the variable amounts of metabolites formed, there were also differences in the type of metabolites formed among individuals. For example, 3,5-diHPV was not observed in some individuals' incubations (i.e., individuals 1, 5, 6, 8, and 14.) which is likely due to a relatively low EGCG clearance rate (i.e., individual 8) or because of the accumulation of the up-

stream precursors EGC and 3,4,5-triHPP-2-ol (i.e., individual 14). For individuals 1, 5 and 6, the non-detectable level of 3,5-diHPV can be either due to a swift further degradation or simply because of the incompetence of the faecal microbiota derived from these individuals in forming the compound.

### 2.3. Quantitative Microbial Profile and Correlation with Metabolite Formation

Figure 5A presents the relative microbial profile at the genus level in the faecal slurries from the 14 individuals (a cut-off of more than 1% of the total bacterial amount in at least one individual was applied). The bacteria fall into 47 genera. Shannon diversity indices of microbial profiles among the 14 individuals ranged between 2.15 and 3.72, indicating that interindividual differences in the microbial relative abundance existed (Figure S4, Supporting Information). Nevertheless, *Firmicutes* and *Bacteroidetes* were consistently found to be the dominant phyla in all samples (Figure S5, Supporting Information). More specifically, except for individual 8, *Faecalibacterium* was found in all individuals in relatively large amounts (15.9% to 40.3% of the total bacteria). In individual 8, *Faecalibacterium* amounted to only 4.9% of the total bacteria. *Bacteroides* was also detected in all individuals, but its level varied strongly between different individuals. For example, the amount was only 2.9% of total bacteria for individual 2 but 66.4% for individual 8. *Prevotella* 9 made up 23.1% to 53.4% of total bacteria in individuals 3, 6, and 9, but did not have a noteworthy contribution in other individuals (Figure 5A). In contrast, the total bacterial load of the 14 individuals showed only small variations (Figure S6, Supporting Information).

To reveal the interplay between the microbial taxonomic profile of the faecal samples of the 14 individuals and their intestinal microbial metabolite profiles, Spearman correlation was performed between the relative abundance of the bacteria (as described above) and the concentrations of residual EGCG plus the concentrations of its seven major metabolites present in the faecal slurry after 2 h of incubation. Statistically significant correlations were found between compound concentrations and bacterial abundance at genus level but not at the phylum level. Figure 5B illustrates the Spearman correlation coefficients at the genus level by a heatmap. The statistical correlation is significant ( $P < 0.05$ ) with  $\rho < -0.53$  or  $\rho > 0.53$ . In total, bacteria from 20 different genera were found to be significantly correlated with at least one of the 8 compounds mentioned above. The concentration of residual EGCG showed an inverse correlation with several genera of the family of *Ruminococcaceae*, which is, *Ruminococcus* 1, *Ruminococcaceae* UCG-005, *Ruminococcaceae*\_uncultured-09, and *Ruminiclostridium* 9, of which the inverse correlation with *Ruminococcaceae* UCG-005 was statistically significant ( $P < 0.01$ ). In addition, also *Bilophila* showed an inverse correlation with residual EGCG ( $P < 0.05$ ). *Ruminococcaceae* UCG-005 was further positively correlated with the concentrations of all seven major metabolites, among which, the correlation with the concentration of gallic acid was statistically significant ( $P < 0.05$ ). *Lachnospiraceae* NK4A136 group and *Lachnospiraceae* UCG-001, which are from the same family of *Lachnospiraceae*, showed similar correlations with seven of the eight compounds.

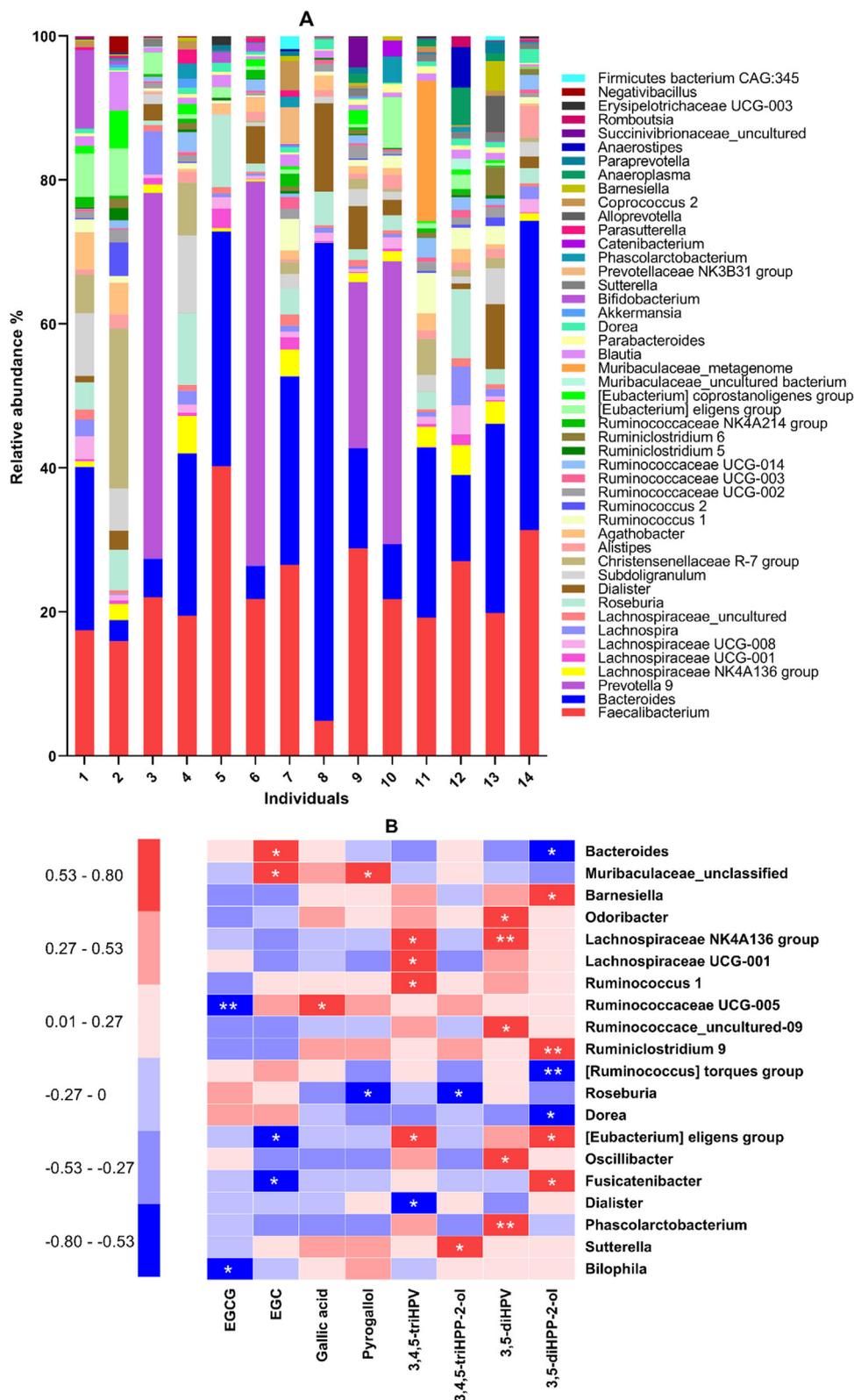


**Figure 4.** EGCG metabolite patterns formed in incubations with faecal slurries from 14 individuals at 2 h. Results are shown as mean  $\pm$  SD from three independent incubations.

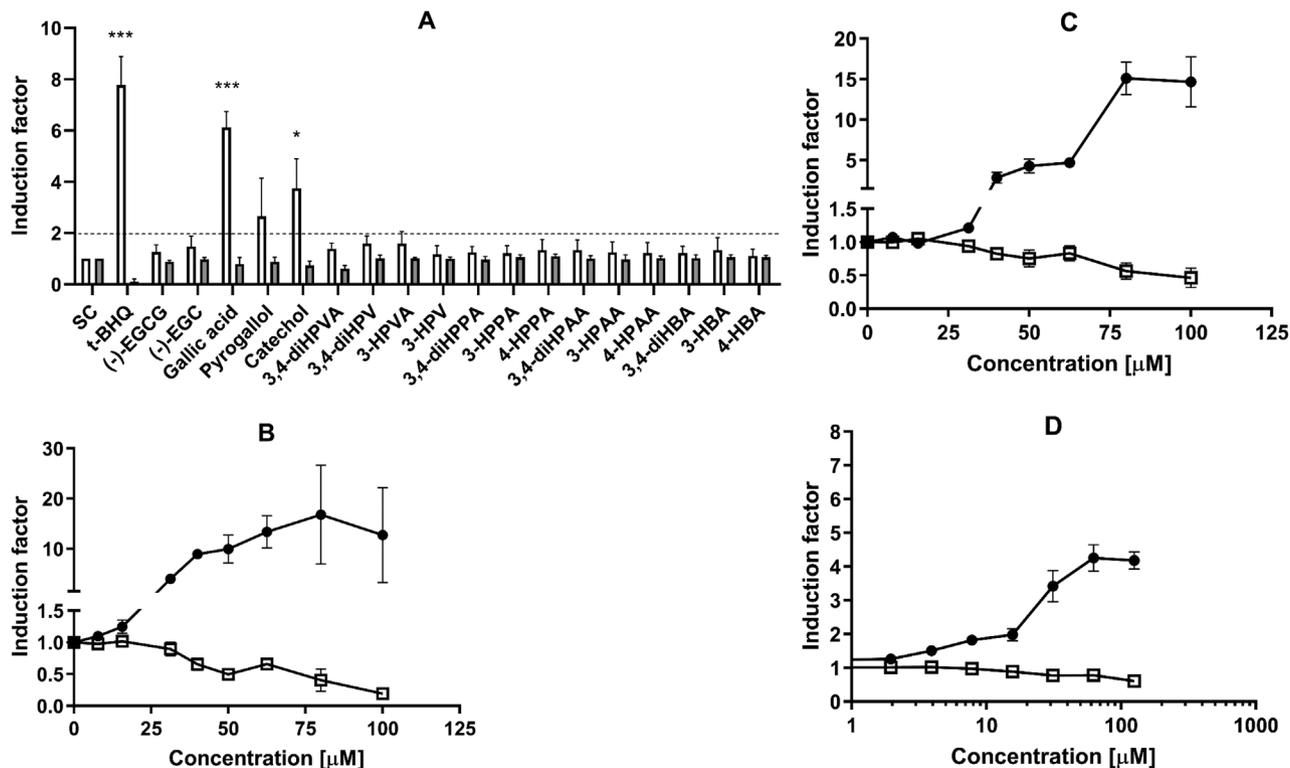
Specifically, both *Lachnospiraceae* NK4A136 group and *Lachnospiraceae* UCG-001 showed statistically positive correlation with the concentration of 3,4,5-triHPV ( $P < 0.05$ ). In addition, *Lachnospiraceae* NK4A136 positively correlated with the concentration of 3,5-diHPV ( $P < 0.01$ ).

#### 2.4. Activation of EpRE-Mediated Luciferase Expression by EGCG and Its Microbial Metabolites

Figure 6A shows the luciferase induction by EGCG and its 17 microbial metabolites at 30  $\mu$ M in the EpRE-LUX assay and the



**Figure 5.** A) Relative microbial abundance at the genus level of the 14 human faecal samples. B) Spearman correlations between the taxon abundances of 20 bacterial genera and the concentration of EGCG and its seven major microbial metabolites present in faecal slurries of 14 individuals after 2 h incubation. The sign and strength of the Spearman correlation coefficients are represented as colors (blue: negative; red: positive). The significances of the correlations are indicated by asterisks: \*  $P < 0.05$ , \*\*  $P < 0.01$ .



**Figure 6.** A) Induction of luciferase activity in EpRE-LUX (white bars) and Cytotox CALUX (grey bar) reporter cells after 24 h exposure to EGCG and 17 EGCG microbial metabolites at 30 µM. The results are presented as mean ± SD of three replicates and the significance of the induction as compared to the solvent control is indicated by asterisks: \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . Induction of luciferase activity in EpRE-LUX reporter cells (closed symbols) and in Cytotox CALUX cells (open symbols) after 24 h exposure to B) gallic acid, C) pyrogallol, and D) catechol at increasing concentrations. Luciferase activity is expressed as induction factor (IF) compared to solvent control and data are presented as mean ± SEM of at least four independent replicates. All IFs were corrected for the viability measured by WST-1 assay showing by Figure S7, Supporting Information.

Cytotox CALUX assay. The induction factors (IFs) obtained in the EpRE-LUX assay were corrected using the respective results from the WST-1 cell viability assay (Figure S7, Supporting Information). Results of the Cytotox CALUX assay show no increase in luciferase activity, ensuring that there was no false positive result in the EpRE-LUX assay due to stabilization of the luciferase reporter protein. The positive control, that is, 30 µM t-BHQ exhibited, a 7.8-fold induction, corroborating the sensitivity of the reporter gene assay. The results reveal that at 30 µM concentration only gallic acid, pyrogallol, and catechol were able to induce EpRE-mediated gene expression by more than twofold compared to the solvent control. Among these three metabolites, gallic acid exhibited the highest efficacy in activating the EpRE-mediated gene expression, with an IF of 6.1. The results obtained reveal that these three microbial metabolites are more potent than the parent compound EGCG. Figure 6B–D presents full concentration-response curves for gallic acid, pyrogallol, and catechol, respectively, enabling definition of the BMCL<sub>5</sub> and BMCU<sub>5</sub> (benchmark concentration), that is, the lower and upper 95% confidence limit of the concentration inducing 5% response. These two values amounted 6.7 and 25.2 µM for gallic acid; 8.0 and 25.3 µM for pyrogallol; 0.1 and 2.9 µM for catechol (Figure S8, Supporting Information). Figure 6B–D also presents the concentration-dependent luciferase activity detected in the Cytotox CALUX reporter cells. No increased luciferase activity was observed in the Cytotox CALUX assay upon exposure to

increasing concentrations of gallic acid, pyrogallol and catechol, again, indicating the absence of luciferase stabilization.

### 3. Discussion

The present paper quantified the time-dependent conversion of EGCG in *in vitro* faecal incubations with pooled and individual human samples in order to characterize the human intestinal microbiota-mediated conversion of EGCG, including the interindividual variability. In addition, EGCG and its microbial metabolites were tested in the EpRE-LUX assay to quantify their potential to induce beneficial EpRE-mediated gene expression.

Using *in vitro* faecal incubations, combined with LC-TQ-MS and LC-TOF-MS analysis, we successfully characterized the time-dependent metabolite profiles of EGCG, identifying seventeen EGCG colonic microbial metabolites and quantifying sixteen of them. The results revealed that gallic acid, pyrogallol, phenylpropane-2-ols, phenyl- $\gamma$ -valerolactones, and 3,5-diHPVA were the major intermediate metabolites formed during the time-dependent human faecal incubations of EGCG. Meanwhile, in the incubations with faecal samples from different individuals, substantial differences in the time-dependent EGCG microbial degradation and the accompanying metabolite patterns were observed among the individuals. Based on the observed differences in the rate of clearance of EGCG, individual 8 was identified as a slow metabolizer. This individual was previously also classified

as a slow metabolizer of the related catechin EC using the in vitro faecal incubation model. Gallic acid and pyrogallol, which were proven to be potent activators for EpRE-mediated genes expression also showed substantial interindividual differences in the amounts produced, amounting to levels between 9.4% and 51.0% and 5.7% to 46.3% of the EGCG equivalents, respectively.

Gallic acid and EGC are the primary metabolites resulting from degalloylation of the D-ring at the 3' position of EGCG. This reaction was reported before to be catalyzed by microbial galloyl esterases present in microbiota, such as *Lactobacillus plantarum* IFPL935, *Enterobacter aerogenes*, *Raoultella planticola*, *Klebsiella pneumoniae* susp. *pneumoniae*, and *Bifidobacterium longum* subsp. *infantis*.<sup>[17,18]</sup> Based on the analysis of the microbial taxonomic abundance of the individual faecal samples, correlations between microbial taxonomic abundance and metabolic abundance were defined in the present study. The results obtained showed statistically significant negative correlations between the quantity of residual EGCG and the relative abundance of *Ruminococcaceae* UCG-005 and *Bilophila* which belong to the phyla *Firmicutes* and *Proteobacteria*. The finding of the latter correlation may point at another phylum that involves in the degalloylation of EGCG besides the ones identified previously. So far, there is no information regarding the enzymes or metabolic pathways responsible for EGCG metabolism in this genus. Perhaps, EGCG or (one of) its metabolites could serve as carbon or energy source or act as a terminal electron acceptor in anaerobic respiration.<sup>[19]</sup>

In line with above observation, *Ruminococcaceae* UCG-005 showed a significant positive correlation with the amount of gallic acid formed. Gallic acid is subsequently decarboxylated by gallate decarboxylase to yield pyrogallol, which can be further metabolized by benzyl alcohol dehydrogenases, leading to the formation of catechol.<sup>[20]</sup> The concentration of gallic acid is inversely correlated with the amounts of *Lachnospiraceae* (although is not statistically significant), suggesting *Lachnospiraceae* is capable of the decarboxylation reaction, which is in agreement with the review written by Cortés-Martín and colleagues.<sup>[21]</sup> Catechol appeared to accumulate only to a limited extent, which hampered its quantification, probably because of its swift further conversion by catechol 1,2-dioxygenases.<sup>[22]</sup> In contrast, relatively large amounts of gallic acid and pyrogallol were detected at multiple time points of the incubation with pooled faecal samples.

Furthermore, the reductive cleavage of the heterocyclic C-ring of EGC resulted in time-dependent increases in the concentration of 3,4,5-triHPP-2-ol. *Eubacterium* SDG-2, *Lactobacillus plantarum*, *Eggerthella lenta* rK3, and *Adlercreutzia equolifaciens* were reported to be able to produce the metabolite phenylpropan-2-ol from catechins.<sup>[18,23,24]</sup> In this study, we found a significant positive correlation between the relative abundance of [*Eubacterium*] *eligens* group and the formation of 3,5-diHPP-2-ol. It was reported that *Flavonifractor plautii*, which belongs to the class of *Clostridia*, was capable of the further metabolism of phenylpropan-2-ol into phenyl- $\gamma$ -valerolactones.<sup>[18,23]</sup> In our study, several genera of *Clostridia* were significantly positively correlated with the formation of 3,4,5-triHPV or 3,5-diHPV, that is, *Lachnospiraceae* NK4A136 group, *Lachnospiraceae* UCG-001, *Ruminococcus* 1, *Ruminococcaceae*\_uncultured-09, [*Eubacterium*] *eligens* group, and *Oscillibacter*. It is of note that studies reported so far in this field focused on the identification of the potential involvement of specific genera in EGCG metabolism by elu-

cidating correlations between bacterial genera and metabolites formed.<sup>[25,26]</sup> However, detailed information on actual enzymes and pathways in the respective intestinal microbiota responsible for the EGCG conversion has not yet been elucidated and provide an interesting topic for future research.

Both 3,5-diHPV and its precursor 3,5-diHPP-2-ol were quantified at higher levels than their isomers 1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-2-propanol (3,4-diHPP-2-ol) and 3,4-diHPV (the latter was identified and quantified with reference standard), respectively, indicating a preference of the intestinal microbiota for performing 4'-dehydroxylation over 5'-dehydroxylation in the B-ring of the molecular skeleton. In the study of van Duynhoven et al. (2014), 3,5-diHPV and 3,4-diHPV were both purified and subsequently identified using Orbitrap MS and 1H NMR, so both isomers can be expected to be present in the microbial conversion of EGCG.<sup>[27]</sup> The latter molecule, 3,4-diHPV, was proposed by van Duynhoven et al. (2014) to be formed primarily from microbial conversion of EC.<sup>[27]</sup> This high level of formation of 3,5-diHPV in EGCG conversion is in line with an in vivo study that reported high human urinary levels (8.3  $\mu\text{M}$ ) of 3,5-diHPV at 9–12 h following an oral dose of 200 mg pure EGCG, suggesting a considerable amount of this compound entered the systemic circulation of human subjects.<sup>[28]</sup>

Also, other characteristics of the in vitro faecal metabolite patterns were consistent with in vivo data. For example, phenyl- $\gamma$ -valerolactones were proven to be among the most abundant metabolites detected in plasma and urine in several in vivo studies in which human volunteers were exposed to green tea or catechin standards.<sup>[10,11,29,30]</sup> Moreover, the major metabolite pyrogallol detected in high abundance in the in vitro incubation was also reported to be one of the main metabolites in urinary excretion after green tea consumption by human volunteers.<sup>[30]</sup> It is worth noting that consumption of black tea could also result in relatively high amounts of gallic acid and pyrogallol detected in vivo.<sup>[31,32]</sup> For instance, Duynhoven et al. (2014) quantified eleven polyphenols and microflora-derived metabolites in plasma after a single-dose black tea extract consumption by human subjects. Among all, pyrogallol-2-O-sulfate was found to be the most abundant conjugated metabolite with a  $C_{\text{max}}$  of 2.6  $\mu\text{M}$  and  $t_{\text{max}}$  of 8 h.<sup>[27]</sup>

There are  $10^{11}$ – $10^{12}$  bacteria per g in the colon content, the highest amount of all parts of the gastrointestinal tract of the host.<sup>[33]</sup> By combining innovative cultivation techniques with high-throughput sequencing techniques, it was shown that up to 95% of molecular species detected in the cecum have corresponding strains in faecal cultures of mammalian gut microbes.<sup>[34,35]</sup> Moreover, use of anaerobic faecal in vitro incubations to study colonic metabolism was previously evaluated in a study in which microbial-related in vivo metabolic changes in gut tissue, cecum content, and feces of rats treated with antibiotics were compared.<sup>[36]</sup> Based on the results obtained it was concluded that "as a non-invasive sampling method, feces provide a suitable matrix for studies on metabolism by the gut microbiota."<sup>[36]</sup>

The beneficial health effects associated with green tea consumption are often ascribed to the tea catechins including EC, ECG, EGC, and EGCG, of which EGCG is the most abundant.<sup>[2]</sup> However, EGCG has been reported to show the lowest absorption of all tea catechins because of its 3-O-galloyl moiety, with a

$C_{\max}$  of 0.12  $\mu\text{M}$  in human plasma upon a single 50-mg dose.<sup>[37]</sup> This concentration of EGCG appears to be unable to activate EpRE-mediated gene expression (Figure 6).<sup>[9,38]</sup> For this reason, in the present study we, investigated whether the microflora-derived EGCG metabolites possess the potential to activate EpRE-mediated gene expression. From the results obtained in the EpRE-LUX reporter gene assay, especially the EGCG metabolites gallic acid, pyrogallol and catechol showed a high capacity for inducing Nrf2 activation. These three compounds have at least two adjacent hydroxyl moieties on their benzene rings, facilitating their (auto)oxidation to (semi)quinones, required to enable activation of EpRE-mediated gene expression.<sup>[39]</sup> In the present study, gallic acid, pyrogallol, and catechol were shown to be able to activate EpRE-mediated gene expression in a concentration-dependent manner with a  $\text{BMCL}_5$  of 6.7, 8.0, and 0.1  $\mu\text{M}$ , respectively, that is, at concentrations that are reported to be physiologically achievable in *in vivo* studies.<sup>[40,41]</sup> The maximum EpRE-mediated induction of luciferase activity by gallic acid and pyrogallol were comparable, with an IF of 16.8 and 15.1, respectively, being substantially higher than that of catechol (IF = 5.0). This might be related to the fact that anti-radical and antioxidant activity, and thus electron donating capacity and potential to generate (semi)quinones were reported to be positively correlated with the number of hydroxyl moieties on the aromatic ring.<sup>[42]</sup> This also explains the absence of EpRE-mediated luciferase induction for monohydroxylated phenolics. Additionally, the high Nrf2-activation induction by gallic acid and pyrogallol also corroborates the importance of the galloyl moiety in their chemoprotective properties.<sup>[38]</sup>

Interestingly, EGCG was reported to be the most potent inducer of EpRE-mediated gene expression compared with other major tea catechins.<sup>[9,38]</sup> We now report that its microbial intestinal metabolites gallic acid, pyrogallol, and catechol are substantially more potent than the parent catechin EGCG and more likely to activate EpRE-mediated luciferase gene expression at physiologically relevant low and even sub $\mu\text{M}$  concentrations. A possible reason for the relatively lower EpRE-mediated activity of EGCG as compared to its degradation products could be that in contrast to gallic acid, pyrogallol, and catechol, EGCG has a bulky 3D structure which may hamper interaction of its quinone type metabolites with the respective cysteine residues in Keap1 and/or its cellular uptake as compared to that of the lower molecular weight phenolics. Given these differences between EGCG and its low molecular weight metabolites it is also of interest to note that after a similar dose of EGCG and gallic acid, the plasma  $C_{\max}$  of gallic acid is 33.3 times the  $C_{\max}$  of EGCG.<sup>[37]</sup> Additionally, gallic acid, pyrogallol, and catechol obtain a planar molecular structure while EGCG is a non-planar chemical. Boerboom, et al. (2006) emphasized the necessity of the planar structure of phenolics in inducing EpRE-mediated gene expression in the EpRE reporter gene assay. They compared EpRE-mediated transcription activation of five planar flavonoids and one non-planar flavonoid, and reported that the lack of the C2-C3 double bond causing a non-planar aliphatic C-ring in taxifolin could be a possible reason for the distinctive unresponsiveness of this non-planar flavonoid in the EpRE-LUX reporter gene assay.<sup>[16]</sup> Moreover, various phenyl-carboxylic acid metabolites were found in the present study to be less potent or even inactive in inducing EpRE-mediated gene expression in spite of the presence of a catechol moiety in their

chemical structure (e.g., 5-(3',4'-dihydroxyphenyl)valeric acid, 3-HPVA, etc.). This is likely due to the fact that at the neutral pH in the EpRE-LUX assay medium, their carboxyl moiety will be largely deprotonated, hampering their diffusion over the cellular membranes. As a result, their low intracellular concentrations may explain their low activity in the bioassay.<sup>[43,44]</sup>

In conclusion, this study introduced an *in vitro* anaerobic incubation model to study microbial metabolism of EGCG. The microflora-derived metabolites were qualitatively and quantitatively detected with the LC-TQ-MS and LC-TOF-MS. Substantial interindividual differences were found in the formation of EGCG gut microbial metabolites both in terms of type and level, which is likely due to the differences in host microbiota composition. EGCG and its major microbial metabolites were subsequently tested in the EpRE-LUX reporter gene assay to compare their potency for Nrf2-activation. Two major metabolites, that is, gallic acid and pyrogallol, together with a minor metabolite catechol, were shown to be potent inducers of EpRE-mediated gene expression. Because these two major metabolites, at physiologically relevant concentrations, showed higher potential for EpRE-mediated gene expression than EGCG, it is concluded that these microbial metabolites may contribute to the potential beneficial effects of EGCG. The interindividual differences in the level of their formation and bioaccumulation may result in interindividual differences in the beneficial effects of EGCG and the potential health-promoting effects of green tea consumption.

#### 4. Experimental Section

**Chemicals and Instruments:** The test compounds were ordered either from Sigma Aldrich (Zwijndrecht, The Netherlands) or Enamine (Kyiv, Ukraine). WST-1 reagent was purchased from Roche (Mannheim, Germany). Alpha-Modified Eagle's Medium ( $\alpha$ -MEM), Dulbecco's Modified Eagle Medium with 1:1 Ham's Nutrient Mixture F-12 (DMEM/F12), penicillin/streptomycin, and phosphate buffered saline (PBS) were purchased from Gibco (Paisley, UK). Nonessential amino acids (NEAA), trypsin, and geneticin (G418) were supplied by Invitrogen Corporation (Breda, The Netherlands). Foetal calf serum (FCS) was obtained from Bodinco (Alkmaar, The Netherlands). Methanol and acetonitrile (ACN) were obtained from Biosolve BV (Valkenswaard, The Netherlands). Acetic acid was ordered from Merck KGaA (Darmstadt, Germany) and formic acid was purchased from VWR CHEMICA (Amsterdam, The Netherlands). The anaerobic chamber used was a BACTRON300 produced by Sheldon manufacturing INC (Cornelius, USA). Para-Pak SpinCon tubes were purchased from Fisher Scientific (New Hampshire, USA).

**Cell Lines:** The EpRE-LUX cells were Hepa-1c1c7 mouse hepatoma cells, stably transfected with a reporter construct carrying a luciferase reporter gene under transcriptional control of an EpRE from the human NAD(P)H: quinone oxidoreductase 1 (NQO1) gene in conjunction with a minimal promoter and an initiator.<sup>[16]</sup> The cells were cultured in  $\alpha$ -MEM supplemented with 10% FCS and penicillin/streptomycin (final concentrations 10 U mL<sup>-1</sup> and 10  $\mu\text{g}$  mL<sup>-1</sup>, respectively) (designated as growth medium).

The Cytotox CALUX cells were human osteosarcoma U2OS cells stably transfected with a reporter construct carrying a luciferase reporter gene under transcriptional control of a constitutive promoter. The reporter construct was generated by inserting the luciferase gene into the multiple cloning site of the pSG5-neo vector.<sup>[45]</sup> These cells have an invariant luciferase expression and were originally designed to discover cytotoxicity.<sup>[46]</sup> The Cytotox CALUX cells were cultured in DMEM/F12 supplemented with 7.5% FCS, 1% NEAA, and penicillin/streptomycin (final concentrations 10 U mL<sup>-1</sup> and 10  $\mu\text{g}$  mL<sup>-1</sup>, respectively). Both cell lines

were maintained at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> and 200 µg mL<sup>-1</sup> G418 was added to the culture medium once a week in order to maintain the selection pressure.

**Faecal Sample Preparation:** Faeces were collected from 14 healthy individuals without dietary restriction. They were of different gender, age, and ethnicity. All of them had not taken antibiotics for at least 3 months prior to the study. The donors consisted of five males and nine females and their ages ranged between 19 and 65. After donations, faecal samples were collected and weighed, and then immediately transferred to the anaerobic operating chamber. The whole process was finished within 5 min to ensure the quality of faecal samples. Subsequently, these faecal samples were diluted with an anaerobic solution of 10% (v/v) glycerol in PBS to get a final faecal concentration of 20% (w/v). After that, samples were filtered using a Para-Pak SpinCon tube and centrifuged at 21 500 × g for 5 min at 4 °C to remove larger particles. The resulting faecal suspension was collected and aliquoted. All the steps mentioned above were carried out under anaerobic conditions. Aliquots of faecal suspension were stored at -80 °C until use. The effect of freezing, storing, and thawing on the microbial metabolism of EGCG by the faecal samples was tested and it was shown not to affect the activity (Figure S1, Supporting Information). The experimental protocol was evaluated and approved to not require further evaluation by the Medical Ethical Reviewing Committee of Wageningen University (METC-WU) based on the Dutch Medical Research Involving Human Subjects Act. All participants gave their written consent.

**Faecal Batch-Culture Incubation:** Faecal incubations were performed in 1.5 mL Eppendorf tubes containing 79% anaerobic PBS, 20% of the above-mentioned faecal slurries (final faecal concentration: 40 mg mL<sup>-1</sup>) and 1% (v/v) of 10 mM EGCG (in methanol) or methanol as control (referred to as "negative control"). The resulting final concentration of EGCG in the solution was 100 µM, which has been shown not to affect the microbiota<sup>[47]</sup> while permitting detection of substrate and metabolites. Incubations of EGCG without faecal slurries were included as blank control. All faecal incubations were carried out in the BACTRON300 anaerobic chamber with an atmosphere of 85% N<sub>2</sub>, 10% CO<sub>2</sub>, and 5% H<sub>2</sub> at 37 °C. Aliquots of incubated faecal samples (50 µL) were collected after 0, 1, 2, 3, 4, 5, 6, and 24 h of incubation for pooled faecal slurries and mixed with 1 volume of ice-cold methanol to terminate the reactions. For studies on interindividual differences, a timepoint of 2 h was selected and incubations were carried out with faecal slurries from each individual. Subsequently, samples were put on ice for at least 15 min, followed by centrifuging at 21 500 × g for 15 min at 4 °C to precipitate proteins, microorganisms, etc. The supernatant of each sample was collected and stored immediately at -80 °C until LC-TQ-MS and LC-TOF-MS analysis for metabolite levels. All the incubations were performed in at least triplicate.

**LC-TQ-MS Analysis:** A Shimadzu LC-TQ-MS 8045 was used to quantify the concentration of EGCG and its metabolites. This equipment consisted of an ultra-high-performance liquid chromatography system coupled to a tandem triple quadrupole mass spectrometer, fitted with an ESI source (Shimadzu, Benelux, B.V. The Netherlands). The ESI source was operated in negative ion mode, and fragment ions *m/z* were obtained. Chromatographic separation was performed on a Waters Acquity UPLC BEH C18 column (2.1 × 50 mm; 1.7 µm) at 40 °C. Solvent A was composed of water: acetic acid (999: 1, v/v), and solvent B was methanol absolute. The eluents were delivered at a flow rate of 0.4 mL min<sup>-1</sup> and the following gradient was used: 0–0.5 min: 5% B, 0.5–5 min: 5–25% B, 5–6 min: 25–100% B, 6–7 min: 100% B, 7–8 min: 100–5% B, 8–13 min: 5% B.

Chromatographic peaks were identified by comparison of the retention time and ion values with those of commercially available authentic standards. The identification information for EGCG and its potential colonic metabolites are summarized in Table S1, Supporting Information. To quantify the metabolites formed during *in vitro* incubations, a set of mixed authentic standard calibrators was used to build calibration curves using fourteen concentrations between 0.01 and 50 µM, and plotting the peak area against the concentration to define the linear regression equation used for quantification. Concentrations of metabolites formed were calculated, including a correction for the amounts detected in corresponding negative controls incubated without adding EGCG. Each experimental replicate was corrected with the mean value of the 3 negative controls.

**LC-TOF-MS Analysis:** An Agilent 1200 series high performance liquid chromatography system coupled with a Bruker micrOTOF (time-of-flight mass analyzer) was used to qualitatively and quantitatively detect pyrogallol and other metabolites whose standard references were not available. Chromatographic separation was performed on the same column as used in the LC-TQ-MS analysis. Solvent A was composed of water: formic acid (999: 1, v/v) and solvent B was ACN: water (999: 1, v/v). The eluents were delivered at a flow rate of 0.2 mL min<sup>-1</sup> and the following gradient was used: 0–2.5 min: 100% A, 2.5–40 min: 0–40% B, 40–45 min: 40–100% B, 45–50 min: 100% B, 50–55 min: 100–0% B, 55–90 min: 100% A. Relative retention time and M-H value were used to identify the aforementioned EGCG colonic metabolites. Specifically, molecules such as 3,4,5-triHPP-2-ol, 1-(3'-hydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-2-propanol and 3,4,5-triHPV have high M-H values (i.e., 307.08, 275.09, 223.06) which are unique in EGCG microbial conversion pathway. Moreover, chemical standards mentioned in LC-TQ-MS were also analyzed in LC-TOF-MS, which helped to annotate compounds sharing the same M-H values. For example, although 3,5-diHPV and 3,4-diHPV have the same M-H values, that is, 207.07, the measurement of the 3,4-diHPV standard in LC-TOF-MS ensured the distinguishing of these two chemicals. Furthermore, previous data from EC microbial conversion samples and time-dependent formation in the pathway were also taken into consideration. For instance, 3,4-diHPP-2-ol was the unique metabolite in EC microbial conversion pathway with a retention time of 19.6 min, which was used to differentiate it from its isomer 3,5-diHPP-2-ol that has a retention time of 18.5 min. Altogether, these ensured the unequivocal identifications of those metabolites for which no standards were available. With the exception of pyrogallol, all the other metabolites were quantified using calibration curves of EC because of the absence of commercial standards. Detailed identification information is summarized in Table S1, Supporting Information.

**Microbial Taxonomic Profiling and Total Bacterial Load:** Aliquots of all 14 faecal samples were sent to an accredited commercial laboratory (IMGM Laboratories GmbH, Martinsried, Germany) for DNA extraction, PCR, library preparation, and sequencing. Additionally, quantification of the bacterial load was carried out by real-time qPCR. PCR products were generated by amplification using 16S V3-V4 primers (F-NXT-Bakt-341F: 5'-CCTACGGGNGGCWGCAG-3' and R-NXT-Bakt-805R: 5'-GACTACHVGGGTATCTAATCC-3'). During an index PCR, barcodes for multiplexed sequencing were introduced using overhang tags. A sequencing library was prepared from barcoded PCR products and sequenced on the Illumina MiSeq next generation sequencing system (Illumina Inc.). Signals were processed to \*.fastq-files and the resulting 2 × 250 bp reads were demultiplexed. Microbiota identification was carried out by clustering the sequences at a 97% identity threshold defining operational taxonomic units (OTU), according to the taxonomy of the SILVA 132 16S rRNA sequence database.

**EpRE-LUX Assay:** The EpRE-mediated induction of gene expression by EGCG and its major intestinal microbial metabolites was tested by measuring the induction of luciferase activity in the EpRE-LUX cells. The assay was performed as described before.<sup>[9,16]</sup> In brief, EpRE-LUX cells were seeded in the inner 60 wells of a white 96-well microplate with a clear bottom (PerkinElmer) at a density of 2 × 10<sup>4</sup> cells per well in 100 µL growth medium. The microplates were incubated at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> for 24 h to allow the cells to form a confluent monolayer. Subsequently, the growth medium was replaced by assay medium (α-MEM without FCS and antibiotics) containing different concentrations of the phenolic test compounds for a continuous 24 h exposure. A total of eight to twelve different concentrations of each compound were tested, ranging from 2 to 125 µM. t-BHQ at 30 µM was used as the positive control in each plate. All test compounds, including t-BHQ, were dissolved in DMSO and added to the cells from 200 times concentrated stock solutions in DMSO. The final concentration of DMSO in the exposure medium was 0.5%. After the 24 h exposure, the assay medium was discarded and the cells were carefully washed and lysed. Subsequently, the microplates were first put on ice for 15 min and then frozen in -80 °C for at least 1 h. Before measuring the luciferase activities of each well using a luminometer (GloMax-Multi Detection System-Promega), the microplates were thawed and raised to room temperature. Relative light units (RLU) were

measured after the addition of 100  $\mu$ L flash mix (20 mM Tricine, 1.07 mM (MgCO<sub>3</sub>)<sub>4</sub>Mg(OH)<sub>2</sub> • 5H<sub>2</sub>O, 2.67 mM MgSO<sub>4</sub>, 0.1 mM EDTA, 2.0 mM DTT, 470  $\mu$ M D-luciferine, 5.0 mM ATP; pH 7.8) into each well. The results are presented as the IF compared to the solvent control. At least three independent biological replicates were performed for all the compounds tested.

**Cytotox CALUX Assay:** To investigate whether stabilization of the luciferase enzyme (false positive) is occurring during the exposure to tested compounds, a parallel Cytotox CALUX assay was performed using U2OS Cytotox CALUX cells that express a constant amount of luciferase.<sup>[48]</sup> This assay was performed in the same way as described above for the EpRE-LUX assay except for the fact that  $1 \times 10^4$  cells per well were seeded in a 96-well microplate and DMEM/F12 was used instead of  $\alpha$ -MEM.<sup>[48]</sup>

**Cell Viability Assay:** To avoid false negative results, EpRE-LUX cell viability was assessed using the WST-1 assay. Briefly, after the same seeding and exposure steps as EpRE-LUX assay, 6  $\mu$ L of WST-1 solution was added to each well of the inner 60 wells of the 96-well microplates. Subsequently, microplates were placed in the incubator at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> for 1 h, followed by measurements of absorbance at 440 and 620 nm by a microplate spectrophotometer. The amount of formazan dye produced by cells was calculated using the values at 440 nm subtracted from the values at 620 nm. The results are presented as viability (%) compared to the solvent control set at 100%.

**Dosage Information:** The concentration of EGCG used in the faecal incubations was 100  $\mu$ M. This concentration will result from an oral dose of  $\approx$ 33 mg per person assuming 52% of the dose would reach the large intestine<sup>[49,50]</sup> and a large intestinal volume of 371 mL for a 70 kg person.<sup>[51]</sup> This dose level would be achieved by drinking 1/3 of a cup of green tea containing 110 mg of EGCG.<sup>[49,50]</sup> The concentrations of catechol, pyrogallol, and gallic acid used in EpRE-LUX reporter gene assay ranged between 2 and 125  $\mu$ M. Based on the assumptions presented above drinking a cup of green tea containing 110 mg EGCG would result in a concentration of EGCG in the large intestine of 337  $\mu$ M. The concentrations of gallic acid and pyrogallol formed in the anaerobic faecal incubations of the present study amounted respectively up to 34% and 32% of the original concentration of EGCG. A similar conversion of 337  $\mu$ M EGCG in the large intestine will result in concentrations up to 115 and 108  $\mu$ M, which are in line with the concentration range tested in the EpRE-LUX reporter gene assay.

**Data Analysis:** LC-TQ-MS data were obtained and processed by Shimadzu Labsolutions software. LC-TOF-MS data were obtained and processed by HyStar software. ChemDraw 18.0 (PerkinElmer, Waltham, USA) was used to draw chemical structures. Graphpad Prism 8.2 (San Diego, USA) was used to plot graphics. Spearman's rank correlation coefficients  $\rho$  ( $-1 \leq \rho \leq 1$ ) were calculated between the concentrations of the eight most relevant metabolites present after 2 h of incubation and the relative abundances of the bacterial genera (range between 0 and 100%). To display the correlations, a heatmap was constructed. The benchmark dose (BMD) approach was applied on the in vitro concentration response curves obtained from the EpRE-LUX assay. The European Food Safety Authority<sup>[52]</sup> BMD modeling web tool was used to calculate BMC according to its manual.<sup>[52]</sup>

## Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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## Conflict of Interest

The authors declare no conflict of interest.

## Author Contributions

C.L. and J.V.D.E. performed experiments. J.V. and L.D.H. provided technical support. C.L. and M.B. interpreted data. C.L. wrote the manuscript. K.B. and I.M.C.M.R. designed the scope of the manuscript and revised the manuscript. All authors read and approved the final manuscript.

## Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article

## Keywords

(-)-epigallocatechin-3-O-gallate, gallic acid, microbial metabolism, Nrf2 activation, pyrogallol

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